NICTABA and UDA, two GlcNAc-binding lectins with unique antiviral activity profiles

SHORT RUNNING TITLE: Antiviral activity profile of NICTABA

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ABSTRACT

Objectives: This study aimed to assess the antiviral properties of a unique lectin (NICTABA) produced by the tobacco plant, *Nicotiana tabacum*.

Methods: Cellular assays were used to investigate the antiviral activity of NICTABA and *Urtica dioica agglutinin* (UDA). Surface plasmon resonance (SPR) studies were performed to study the sugar specificity and the interactions of both lectins with the envelope glycoproteins of HIV-1.

Results: The GlcNAc-binding lectins exhibited broad-spectrum activity against several families of enveloped viruses including influenza A/B, DENV2, HSV-1/2 and HIV-1/2. The IC_{50} of NICTABA for various HIV-1 strains, clinical isolates and HIV-2 assessed in PBMCs ranged from 5 to 30 nM. Furthermore, NICTABA inhibited syncytium formation between persistently HIV-1-infected T-cells and uninfected $CD4^+$ T lymphocytes and prevented DC-SIGN-mediated HIV-1 transmission to $CD4^+$ target T lymphocytes. However, unlike many other antiviral carbohydrate-binding agents (CBAs) described so far, NICTABA did not block HIV-1 capture to DC-$SIGN^+$ cells and it did not interfere with the binding of the human mAb 2G12 to gp120. SPR studies with HIV-1 envelope glycoproteins showed that the affinity of NICTABA for gp120 and gp41 was in the low nanomolar range. Specific binding of NICTABA to gp120 could be prevented in the presence of a GlcNAc trimer but not of mannose trimers. NICTABA displayed no antiviral activity to non-enveloped viruses.

Conclusions: Since CBAs possess a high genetic barrier for the development of viral resistance and NICTABA's broad antiviral activity profile, this CBA may qualify as a potential antiviral candidate with a pleiotropic mode of action aimed at targeting the entry of enveloped viruses.

Keywords:

NICTABA, UDA, GlcNAc, glycosylation, broad spectrum, antiviral peptide

INTRODUCTION

Viruses such as immunodeficiency virus (HIV), herpes simplex virus (HSV), influenza virus and hepatitis C virus (HCV) contain highly glycosylated proteins in their envelope. The HIV envelope protein gp120 is among the most heavily glycosylated proteins with almost 50% of its molecular weight being due to the presence of N-linked glycans.[1,2] These glycans play an important role in the conformation of the envelope during synthesis and in viral transmission and subsequent infection.[3-7] They also function as a shield to reduce the accessibility of the immunogenic protein core in order to prevent an efficient immune response.[8-11] Given the important structural and functional role of the viral envelope glycans, various carbohydrate-binding agents (CBAs) have been investigated for their potential to interfere with virus infection. In fact, CBAs have been proposed as a novel interesting class of antiviral drugs since they have been shown to have the capacity to inhibit (*i*) infection of cells by cell-free virus particles, (*ii*) the interaction between HIV-infected leukocytes and uninfected $CD4^+$ cells, but also *(iii)* the capture of virus by DC-SIGN-expressing dendritic cells and macrophage mannose receptor (MMR)-expressing macrophages[12,13] and (*iv*) transmission of such captured virus to uninfected $CD4^+$ T lymphocytes.[14]

Because these agents block both entry and transmission of the virus, they would qualify as potent antiviral compounds with a pleiotropic mode of action. Several CBAs have therefore been studied more in detail for their antiviral properties.[12,15-24] It has been shown that apart from their ability to prevent virus infection, capture and transmission, they are also able to force the virus to delete N-glycosylation sites in their envelope after prolonged drug exposure. Indeed, after prolonged exposure of HIV-1 gp120 to escalating doses of a broad variety of CBAs (*i.e.*, HHA, GNA, CV-N, 2G12, AH, MVN, PRM-S, GRFT or UDA), up to eight or nine N-glycosylation sites were deleted in the envelope of such mutant virus strains.[25-29] This implies a high genetic barrier for CBA treatment of viral infections since often more than 3 to 5 N-glycan deletions in the HIV gp120 are required to afford a significant phenotypic resistance to CBAs. Also, the uncovering of previously hidden immunogenic epitopes on the surface of the virion by the glycan deletions may trigger an immune response against these epitopes. It has indeed been shown that mutant HIV-1 strains that have several N-glycan deletions in their envelope, are more prone to neutralizing antibodies directed against gp120.[11,14,30] For this reason the CBAs display a unique combination of antiviral mechanisms. A specific subgroup of CBAs are lectins. Although lectins from natural origin have garnered much interest due to their specific interaction with carbohydrates and their strong antiviral properties, many hurdles have hampered the translation of their preclinical success. Firstly, lectins as a therapeutic strategy must overcome the issues posed by their protein nature, more specifically their sensitivity to proteolytic degradation, their large size, their short half-live and potential immunogenicity. Secondly, large-scale production and purification of these natural products is very costly and technically challenging. However, computational methods (*e.g.*, EpiSweep) are currently available that aid in the design of immunotolerant therapeutic proteins with preserved structural properties.[31] Furthermore, low molecular weight synthetic compounds with lectin-like properties could be an alternative solution for these problems. Pradimicin-A, pradimicin-S and benanomicin-A have also shown to have antiviral activities and represent the first prototype drugs of non-peptidic low-molecular-weight CBAs.[32-34] These compounds may be good candidate CBAs for further preclinical research. Although peptidic lectins are currently not being evaluated in clinical trials, they should nevertheless be considered as valuable preclinical screening tools to find novel drug candidates that mimic their activity.

UDA is a chitin-binding protein from the hevein family. It adopts a monomeric form in solution and is isolated from the rhizome of the stinging nettle (*Urtica dioica,* UDA) as a complex mixture of isolectins.[35] With its 8.5 kDa molecular weight, it is one of the smallest lectins reported and it has specific GlcNAc-binding activity.[36] Two hevein domains of 43 amino acids are connected with a short linker of 4 amino acids in the UDA molecule. Through binding studies with GlcNAc oligomers it has been determined that there are presumably three sugar binding areas in both hevein domains meaning that a trimer of GlcNAc is the actual substrate of the binding site. Although the presence of a second binding site on the protein has been postulated by several groups, the affinity of this site for glycans would be more than ten-fold lower than the affinity of the first binding site.[37,38] More recent studies have revealed that UDA isolectin I can form dimers mediated by two Zn^{2+} ions bound to the sugar binding site.[39] UDA is known to be an immunomodulatory agent with insecticidal and fungistatic properties.[40] Earlier, this agent was shown to possess pronounced activity against viruses such as HIV,[41] HCV[42] and human cytomegalovirus (CMV),[41] with a low agglutination activity against human red blood cells.[25,41]

We here focus on the tobacco agglutinin or NICTABA, another GlcNAc-specific CBA, isolated from the plant *Nicotiana tabacum*.[43] This lectin turned out to be a cytoplasmic/nuclear chito-oligosaccharide-binding protein whose expression is induced in tobacco leaves by jasmonates or stress situations such as insect attacks. It forms a homodimer consisting of two subunits with a dimer molecular weight of approximately 38 kDa. The lectin shows a strong affinity for N-acetyl-D-glucosamine

(GlcNAc)–oligomers, but in addition also to high-mannose-type N-glycans.[44] In this study the broad antiviral properties of UDA and NICTABA are compared and their mechanism of HIV-envelope interaction investigated.

MATERIALS AND METHODS

Compounds

The chitin-binding lectin from stinging nettle (*Urtica dioica*, UDA) was purified using a combination of cation exchange chromatography and affinity chromatography on a chitin column, as described previously.[35] The tobacco (*Nicotiana tabacum cv Samsun NN*) lectin NICTABA was purified from tobacco leaves treated with methyl jasmonate using affinity chromatography on chitin followed by anion exchange chromatography as described by Chen *et al.*[43] The mannose-specific lectin *Hippeastrum hybrid* (HHA) was purified from Hippeastrum hybrid bulbs using affinity chromatography on mannose-sepharose, as described previously.[45] The 2G12 mAb was generously provided by Polymun Scientific (Vienna, Austria).

Cell line cultures and primary leukocytes

Human T-lymphocytic C8166, HUT-78, SupT1 and CEM cells were obtained from the American Type Culture Collection (Manassas, VA). MT-4 cells were provided by Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France). The Raji DC-SIGN-expressing Raji/DC-SIGN cells and the TZM-bl cells[46] were kindly provided by Dr. L. Burleigh (Institut Pasteur, Paris, France) and by Dr. G. Van Ham (ITG, Antwerp, Belgium), respectively. The CrFKs were obtained from Dr. H. Egberink, (University of Utrecht, Netherlands). Buffy coat preparations from healthy donors were obtained from the Blood Bank (Red Cross) in Leuven, Belgium. Peripheral blood mononuclear cells (PBMCs) were activated with phytohaemagglutinin (PHA) at 2 µg/mL (Sigma, Bornem, Belgium) for 3 days at 37°C before further use in antiviral assays as PHA-activated PBMCs. All cell lines mentioned were cultivated in

RPMI-1640 medium supplemented with 10% FBS (BioWittaker Europe, Verviers, Belgium), 2 mM L-glutamine and 0.075 M $NaHCO_3$.

HIV strains

HIV-1 (IIIB) was provided by Dr. R.C. Gallo and Dr. M. Popovic (Institute of Human Virology, University of Maryland, Baltimore, MD). HIV-2 (ROD) was provided by Prof. L. Montagnier (at that time at the Pasteur Institute, Paris, France). The primary clinical isolates representing different HIV-1 clades and HIV-2 isolates were all kindly provided by Dr. J. Lathey from BBI Biotech Research Laboratories, Inc., Gaithersburg, MD, and their co-receptor use (R5 or X4) was determined in our laboratory.[47]

Antiviral assays

The antiviral assays were based on the inhibition of virus-induced cytopathicity in confluent cell cultures, and the cytostatic assays on inhibition of cell proliferation in exponentially growing cell cultures according to previously described methodology.[48,49] The antiviral assays, other than the anti-HIV assays, were based on the inhibition of virus-induced cytopathicity in HEL [(HSV-1) (KOS), HSV-2 (G), Vero (parainfluenza-3), HeLa (coxsackie virus B4, reovirus-1 and respiratory syncytial virus), CrFK (feline coronavirus and herpes virus) or MDCK [influenza A (H1N1, H3N2) and influenza B] cell cultures. Confluent cell cultures (or nearly confluent for MDCK cells) in microtiter 96-well plates were inoculated with 100 times the median cell culture infective dose (100 $CCID_{50}$) of virus, and the cell cultures were incubated in the presence of varying concentrations of the compounds. Viral CPE was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the compounds. The minimal cytotoxic concentration (MCC) of the compounds was defined as the compound concentration that caused a microscopically visible alteration of the cell morphology. For the antiviral assay with dengue virus (DENV), the Raji/DC-SIGN$^+$ cells were infected with DENV serotype 2 in absence or presence of the CBAs and analyzed by flow cytometry 4 days post-infection, as described previously.[50]

Anti-HIV replication assays

The methodology of the anti-HIV assays was as follows: human CEM (~ 3 x 10^5 cells/cm^3) cells were infected with 100 $CCID_{50}$ of HIV-1 (IIIB) and seeded in 200 µL wells of a microtiter plate containing appropriate dilutions of the lectins. After 4 days of incubation at 37 °C, HIV-induced CEM giant cell formation was examined microscopically. The antiretroviral assays in MT-4 cells and T-cell blasts have been described in detail earlier.[51] Briefly, MT-4 cells (1 x 10^6 cells/mL) were pre-incubated for 30 minutes at 37°C with the test compounds in a 96-well plate. Next, NL4.3 virus was added according to the $CCID_{50}$ of the viral stock. CPE was scored microscopically 5 days post-infection, and the 50% effective concentration (EC_{50})-values were determined using the MTS/phenazine ethosulfate (PES) method.[51]

The PHA-stimulated blasts were seeded at 0.5 x 10^6 cells per well into a 48-well plate containing varying concentrations of compound in medium containing interleukin (IL)-2 (25 U/mL, R&D Systems Europe, Abingdon, UK). After 30 minutes of pre-incubation with the test compounds, 1,000 pg/mL virus stock (HIV-1, HIV-2, clinical isolates) was added. At day 3 and 6 post-infection 2 ng/mL IL-2 was once more supplemented to the cells. Cell supernatant was collected at day 10 and HIV-1 core antigen (Ag) in the culture supernatant was analysed by a p24 Ag ELISA kit (Perkin

Elmer, Zaventem, Belgium) according to manufacturers' guidelines. For HIV-2 p27 Ag detection the INNOTEST from Innogenetics (Temse, Belgium) was used.

TZM-bl cells were seeded at a density of 17 x 10^4 cells/well in a 96-well plate and were pre-incubated with varying concentrations of compounds at 37°C. After the pre-incubation step, stock of BaL virus was added at a final concentration of 500 pg/mL and incubated for an additional 48 hrs. Luminescence was measured after 10 min incubation with Steadylite Plus Reagent (Perkin Elmer). To determine loss in cell viability, luminescence from the sample-treated wells were compared to that of the cell controls. EC_{50} is reported as the concentration of the sample which reduced the relative luminescence units (RLUs) by 50% compared to the virus control.

Effect of NICTABA or UDA on giant cell formation in co-cultures of Sup T1 cells with persistently HIV-1-infected HUT-78/HIV-1 cells (Giant cell assay).

Persistently HIV-1-infected HUT-78 cell cultures were generated as described previously.[24] For the co-cultivation assays HUT-78/HIV-1 cells were thoroughly washed to remove free virus from the culture medium, and 5 x 10^4 cells (50 µL) were transferred to 96-well plates. Then, a comparable amount of Sup T1 cells (50 µL), along with an appropriate concentration of test compound (100 µL), was added to each well. After 2 days, the EC_{50}-values were determined microscopically based on the appearance of syncytia in the cell co-cultures.

Effect of short exposure of HIV-1 to test compounds on virus capture by Raji/DC-SIGN cells (HIV-1 capture assay).

High amounts of HIV-1 particles (100 µL; 2.2 x 10^6 pg p24/mL) were exposed to serial dilutions of the compounds (400 µL) for 30 min as described before.[13] Then, the drug-exposed virus suspensions (500 µL) were mixed with Raji/DC-SIGN$^+$ cell suspensions (500 µL; 10^6 cells) for 60 min at 37 °C after which the cells were thoroughly washed twice with 40 mL culture medium as described above. This procedure resulted in a final dilution of the initial compound concentrations by at least 160,000-fold. The Raji/DC-SIGN$^+$ cell cultures were then analysed for p24 Ag content.

Exposure of DC-SIGN$^+$ cells to HIV-1 and subsequent co-cultivation with CD4$^+$ T cells (HIV-1 Transmission assay).

The B-lymphocyte Raji DC-SIGN-expressing (Raji/DC-SIGN$^+$) cells were suspended in cell culture medium at 6 x 10^6 cells/400 µL as described by Balzarini *et al.*[13] Briefly, 0.4 mL-cell suspensions were exposed to 600 µL wild-type HIV-1 (IIIB or HE) (2.2 x 10^6 pg/mL p24) for 60 min, after which the cell-virus suspension was 40-fold diluted in culture medium. After centrifugation at 1,250 rpm for 10 min, the obtained pellet was resuspended in a large volume of medium. Following a second centrifugation step, supernatant was once more removed and the remaining 0.1 mL cell suspension was 10-fold diluted in cell culture medium. Under these experimental (washing) conditions, a maximum of 8 pg HIV-1 p24 could have remained in the 1 mL-supernatant (or 0.4 pg in 50 µL). A 50 µL cell suspension was withdrawn for p24 Ag determination by a specific HIV-1 p24 ELISA and 50 µL of the Raji/DC-SIGN$^+$ cell suspension was added to 96-well microplates in which 100 µL compound dilutions were present. Then, 50 µL C8166 cells (10^7/mL) were added to each well. These mixed cell cultures were incubated at 37°C in a CO_2-controlled humidified incubator, and microscopically scored for syncytia formation at ~ 36 to 48 hrs post virus-exposure/co-cultivation. It should be mentioned that the maximum amount of free viral particles that could have remained in the culture medium after extended washing steps (< 1 pg HIV-1 p24) is unable to result in HIV-1-induced giant cell formation in $CD4^+$ T cell cultures within the time period of analysis (36 to 48 hours).

2G12 mAb binding assay.

$CD4^+$ MT-4 cells were infected with HIV-1 NL4.3 and analysed when the CPE started to occur (3-4 days post-infection). Shortly, after washing with PBS supplemented with 2% FCS (PBS/FCS2%), the cells were pre-incubated with various concentrations of NICTABA, HHA or UDA for 30 minutes, extensively washed and incubated with 2G12 mAb for 30 minutes. Afterwards, the cells were washed and further incubated with RaH-IgG-FITC (Dako) for 30 minutes. As a control for non-specific background staining, the cells were stained in parallel with RaH-IgG-FITC only. After washing, the cells were fixed with a 1% aqueous formaldehyde solution and analysed with a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences, San Jose, CA, USA). For the calculations of the mAb binding, the mean fluorescence intensity (MFI) of each sample was expressed as percentage of the MFI of control cells (after subtracting the MFI of the background staining).

Surface Plasmon Resonance

1) Kinetic experiments

For the HIV envelope binding experiments, the recombinant glycoprotein gp120 from the HIV-1 IIIB strain (ImmunoDiagnostics Inc., Woburn, MA; produced in cell cultures of chinese hamster ovaries) and the recombinant glycoprotein gp41 from the HIV-1 HxB2 strain (Acris Antibodies GmbH, Herford, Germany; produced in *P. pastoris* cells) were covalently coupled to the carboxymethylated dextran matrix-covered CM5 sensor chips at flow channels two and four, respectively, (GE

Healthcare, Uppsala, Sweden) in 10 mM sodium acetate, pH 4.0 using the standard amine coupling chemistry affording a final density of 148 RUs for gp120 (1.25 fmol) and 265 RUs for gp41 (6.5 fmol). The flow cells in channels one and three were used as controls for non-specific binding and refractive index changes.

All interaction studies were performed at 25 °C on a Biacore T100 instrument (GE Healthcare, Uppsala, Sweden). The compounds were diluted in HBS-P (10 mM HEPES, 150 mM NaCl and 0,05% surfactant P20; pH 7.4) supplemented with 10 mM Ca^{2+}, using two-fold dilution steps. UDA and NICTABA were used at concentrations ranging from 1.0 to 63 nM. Samples were injected during 3 minutes at a flow rate of 30 μL/min followed by a dissociation phase of 5 minutes. The sensor chip was regenerated with 50 mM NaOH. Several blanks were included throughout the experiment as a second reference.

For the determination of the kinetic parameters, the curves were fitted using the 1:1 binding model (Biacore T100 Evaluation software 2.0.1).

2) *Inhibition of the binding by sugars*

The sensor surface that was prepared in 1) was used to analyse the binding of UDA and NICTABA in the presence or absence of a GlNAc trimer (GlcNAc β-(1,4)-GlcNAc/β-(1,4) GlcNAc) (Dextra Laboratories Ltd, Reading, UK), a mannose/α-(1,2)- mannose/α-(1,2) mannose trisaccharide (Carbohydrate Synthesis, Oxford, UK) or a mannose/α-(1,3)-mannose/α-(1,6)-mannose trisaccharide (Dextra Laboratories Ltd, Reading, UK). The concentration of UDA was kept constant at 100 nM throughout the whole experiment. NICTABA was used at a concentration of 80 nM. The concentrations of the trisaccharides were 200 or 100 μM. The lectins, trisaccharides or a pre-incubated mixture of lectin and trisaccharide were injected across the sensor surface as described for the kinetic experiments.

RESULTS

Broad antiviral activity profile of NICTABA and UDA in cell cultures

NICTABA and UDA have been evaluated for their inhibitory activity against a wide variety of enveloped DNA and RNA viruses in cell culture using the α(1,3)-α(1,6)-mannose-specific HHA as a control (Table 1). Whereas HHA was only inhibitory against influenza A and B viruses, Dengue virus serotype 2 (DENV-2), and HIV (Table 2), the GlcNAc-specific CBAs displayed a much broader antiviral spectrum. They not only inhibited the same viruses as HHA did, but in addition, they also showed inhibitory activity against HSV type 1 (HSV-1) and HSV-2, varicella-zoster virus (VZV) and parainfluenza virus. NICTABA often showed an activity superior to UDA. Interestingly, respiratory syncytial virus (RSV) and HSV-1 were only suppressed by NICTABA but not by UDA (Table 1). None of the three CBAs displayed antiviral activity to non-enveloped viruses such as coxsackie virus and reovirus. The median cytotoxic concentration (CC_{50}-)value of NICTABA in HEL cells was >50 µg/mL (> 1.5 µM).

Broad-spectrum anti-HIV activity profile of NICTABA and UDA

Our first set of experiments demonstrated a broad activity spectrum of the CBAs against enveloped viruses, which are heavily glycosylated. Since HIV is one of the most glycosylated viruses, we evaluated the antiviral activity of CBAs and their mode of action against different HIV strains.

In this part of the study, we measured the activity of NICTABA and UDA against cell line-adapted HIV-1 strains (NL4.3, BaL, IIIB) in different cell models including the genital epithelial cells TZM-bl but also susceptible $CD4^+$ T cell lines such as MT-4 and CEM cells. As a reference we compared the activity of the GlcNAc-binding lectins with the mannose-specific CBA HHA. As shown in Table 2, both NICTABA and UDA could inhibit infection of these HIV strains with an EC_{50} in the lower µM-range (EC_{50}: 0.023 µM – 0.28 µM).

Secondly, the anti-HIV activity of NICTABA and UDA was tested against clade B HIV-1 strains and HIV-2 in PHA-activated PBMCs (or T cell blasts). We found that both CBAs inhibited infection of the CXCR4-using (X4) NL4.3, the CCR5-using (R5) BaL and the dual-tropic HE HIV-1 strains in the lower nM range (IC_{50} NICTABA: 14 nM – 18 nM). Moreover, NICTABA and UDA prevented HIV-2 infection of PBMCs with a mean EC_{50} of 5 and 60 nM, respectively. Overall, the mannose-specific lectin HHA showed a better antiviral activity against HIV-1 laboratory strains compared to both GlcNAc-binding CBAs, but NICTABA demonstrated less variability in its virus-suppressive potential and was 10- to 100-fold more potent than UDA (Table 2).

Thirdly, primary HIV-1 clinical isolates representing different HIV-1 clades (A, B, C, and A/E) were tested for their susceptibility to neutralization by NICTABA and UDA in PBMCs. Both CBAs were found to be protective but the neutralization activity of NICTABA was higher than that of UDA with EC_{50}s varying from 8 to 30 nM. In addition, the antiviral activity of NICTABA was evaluated in monocyte-derived macrophages (MDM) against the HIV-1 BaL strain and was found to be protective with a mean EC_{50} of 0.016 ± 0.004 µM (data not shown). The CC_{50}-value for NICTABA obtained in PBMCs was 0.38 µM. We can conclude that NICTABA and UDA exhibited a consistent and broad anti-HIV activity in both $CD4^+$ T-lymphoma cell cultures and in PBMCs (Table 2). In all cases, the antiviral potency of the GlcNAc-binding agent NICTABA was superior to that of UDA.

NICTABA and UDA inhibit HIV induced cell-cell syncytium formation

When NICTABA and UDA were examined for their inhibitory potential to prevent syncytium formation between persistently HIV-1 IIIB-infected cells (HUT-78/IIIB) and uninfected $CD4^+$ SupT1 cells, both CBAs inhibited giant cell formation to a similar extent (IC_{50}s: 0.32-0.48 μM) but inferior to the mannose-specific HHA (0.08 μM) (Table 3).

NICTABA and UDA have also been investigated for their potential to prevent HIV-1 capture by DC-SIGN-expressing Raji/DC-$SIGN^+$ cells and to block transmission of DC-SIGN-captured virus to $CD4^+$ T-lymphocyte C8166 cell cultures. Whereas NICTABA could not prevent virus capture by Raji/DC-$SIGN^+$ cells, UDA could do so. However HHA was much more efficient than UDA in preventing virus capture (Table 4). On the other hand, transmission of HIV-1-captured virus by Raji/DC-$SIGN^+$ cells to the uninfected $CD4^+$ T cells was dose-dependently inhibited by both NICTABA and UDA (Table 4).

Effect of NICTABA, UDA and HHA on the binding of the carbohydrate binding mAb 2G12 to HIV-1 infected MT-4 cells.

NICTABA at the highest concentration tested (0.52 μM; 20 μg/mL) had no effect on the binding of the carbohydrate-binding mAb 2G12 to HIV-1-infected MT-4 cells, while HHA (0.4 μM; 20 μg/mL) and UDA (2.4 μM; 20 μg/mL) efficiently inhibited this process (Figure 1). Thus NICTABA, in contrast to all the other CBAs examined so far including the mAb 2G12, does not interact with the 2G12 mAb binding epitope on gp120.

In addition, NICTABA, but also the other CBAs HHA and UDA at similar concentrations, had no effect on the binding of the mAb (clone 9205) recognizing the V3 loop of gp120, to HIV-1-infected MT-4 cells (data not shown).

Kinetic interactions of NICTABA and UDA with the HIV envelope glycoproteins gp120 and gp41.

The binding of NICTABA and UDA to the virus envelope was kinetically characterized by means of surface plasmon resonance (SPR) analysis. In this experiment, gp120 and gp41 were covalently immobilised on a CM5 sensor chip. The CBAs UDA and NICTABA were used as analytes in a concentration range of 1.8 to 63 nM for NICTABA, 2.0 to 63 nM (gp120) or 2.0 to 31 nM (gp41) for UDA. The analyte solutions were injected across the sensor chip for 3 minutes after which the analyte was allowed to dissociate from the surface during 5 minutes. The residual analyte was then removed from the surface by using a pulse of 50 mM NaOH. The binding curves of NICTABA and UDA to gp120, together with the fitted curves, are shown in Figure 2A.

The kinetic parameters that were determined for the binding of NICTABA and UDA to gp120 and gp41 are summarized in Table 5. Both CBAs showed K_D (affinity)-values for HIV-1 gp120 in the lower nanomolar range (K_D: 4-9 nM). Whereas the on-rates (k_a) to gp120 were fairly comparable for UDA and NICTABA, the dissociation rate constant (k_d) for NICTABA was 6-fold lower than for UDA.

Their affinities for gp41 were at least as good as, if not better than for gp120 (Figure 2B, Table 5). Comparable apparent K_D-values (1.5-1.6 nM) were detected for the binding of both CBAs to gp41. For UDA the K_D value was 5.4-fold lower (higher affinity) for gp41 than for gp120 (Table 5).

Overall, the sensorgrams show that both CBAs have a high affinity for the envelope proteins gp120 and gp41 with K_D values ranking in the subnanomolar range.

Effect of carbohydrate oligomers on the binding of NICTABA and UDA to HIV-1 gp120.

To verify the nature of the sugar specificity of both lectins, the binding capacity of NICTABA and UDA to immobilised gp120 was investigated in the presence or absence of different glycan structures such as a GlcNAc β-(1,4)/GlcNAc/β-(1,4) GlcNAc, a mannose α-(1,2)-mannose/α-(1,2)-mannose or a mannose/α-(1,3)-mannose/α-(1,6)-mannose oligosaccharide.

Prior to the gp120 binding, the CBAs were incubated in the presence of the respective trisaccharides (trisaccharides at a final concentration of 100 or 200 μM) for a short time period. The GlcNAc trimer and the α-(1,2)/α-(1,2) mannose and the α-(1,3)/α-(1,6) mannose trimers were also injected in the absence of the CBAs at a concentration of 200 μM as a reference. The final concentrations of NICTABA and UDA in these experiments were 80 and 100 nM, respectively. The results are shown in Figure 3.

The binding of NICTABA to gp120 was dose-dependently reduced by the addition of the GlcNAc trimer to the NICTABA solution, while the other two mannose-trisaccharides only had a minor, if any, effect on the binding of NICTABA to immobilised gp120 (Figure 3A). Instead, the binding of UDA to gp120 could be reduced by adding either one of the trisaccharides, although the highest level of inhibition could be observed with the trimer of GlcNAc. Clearly, both mannose trimers interfered with the binding of UDA to gp120 although to a lesser extent compared to the effect of GlcNAc trimers on UDA binding (Figure 3B).

DISCUSSION

Many viruses affecting human health use the host cell machinery to assemble glycans on their surface, which are important for viral entry and escape from the host immune system.[8,9,52] Glycans are bound by natural ligands called lectins that have differential binding preferences for them. Targeting viral glycans is a promising antiviral strategy since there are multiple glycosylated sites on the virus capsid.[53,54] In addition, emergence of resistance to such anti-carbohydrate therapies will most likely involve the removal of multiple sugar residues from the viral envelope thereby more efficiently exposing the surface of the virus to host-derived neutralizing antibodies.[25,53,55-58] Moreover, Balzarini *et al.*[25] demonstrated that the kinetics of resistance development also likely depend on the type of glycan targeted as it took a 3-fold longer time before phenotypic resistance became apparent against UDA, a GlcNAc-binding CBA, than against mannose-specific CBAs. Here, we evaluated the activity of a tobacco plant lectin called NICTABA that has exclusive affinity for GlcNAc residues and compared it with UDA and the mannose-specific HHA.

We found that NICTABA and UDA are both inhibitory against a very broad spectrum of enveloped viruses. Interestingly, this spectrum is much broader than that of HHA. This may be due to the fact that GlcNAc is more abundantly present in complex-type than in high-mannose-type glycans. As HIV is one of the most heavily glycosylated viruses,[59,60] we evaluated the activity of NICTABA, UDA and HHA to a variety of HIV strains in different target cells. Indeed, Raska *et al.*[61] reported that the glycosylation pattern is profoundly influenced by the cell type and metabolic activity of the producing cells, resulting in distinct gp120 N-glycan contents and heterogeneity. While such high variability of the HIV envelope compromises the efficacy of neutralizing antibodies (that only recognize one specific envelope epitope),[62] GlcNAc-specific CBAs maintained their antiviral activity with EC_{50}-values ranking between the lower nano- and micromolar ranges. Furthermore, NICTABA and UDA were endowed with much less variation in their antiviral spectrum than HHA. An important contributing factor to these differences may again be the sugar specificity of these CBAs. While HHA predominantly recognizes α-(1,3)- and/or α-(1,6)-mannose residues located at the pentasaccharide core, GlcNAc oligomers are invariably present at the base of the pentasaccharide core in each N-glycosylation site of gp120. Also, there was a clear tendency for NICTABA to be more potent in inhibiting virus replication than UDA. This may be related to a more pronounced binding of NICTABA to the viral envelope glycans than UDA. Indeed, the apparent affinity of NICTABA for HIV-1 gp120 was 2-fold higher than of UDA, but, importantly, this was mainly due to the contribution of the off-rate, which was ~6-fold lower for NICTABA than UDA.

The affinity measurements between gp120 and NICTABA or UDA showed low K_D values being in the low to sub-nanomolar range. Since the dissociation rates of these CBAs were very low, they can be virtually considered to be irreversible gp120 binders. These tight-binding properties are in agreement with the increased antiviral activities upon pre-incubation of HIV-1 or HIV-1-infected cells with NICTABA and UDA. However, Doores *et al* found that the glycan composition of recombinant monomeric gp120 mainly comprises complex glycans while the envelope proteins of intact virions almost exclusively carry oligomannose glycans.[63] This difference in composition may affect the binding capacity of CBAs and therefore our comparative binding studies performed with recombinant monomeric gp120 needs to be interpreted with some caution and in close connection with the antiviral cell culture data. It was interesting to notice that the affinity of the CBAs for gp41

(K_D: ±1.5 nM) was higher than for gp120 (K_D: 3.8-8.7 nM) while the envelope protein gp120 contains 6-fold more sites for N-linked glycosylation than gp41, which typically contains only 4 sites for N-glycan attachment.[64]

Many viruses including HIV,[65] RSV,[66] and VZV[67] induce membrane fusion between cells to form giant multinucleated cells, called syncytia, which allows the virion to kill many cells by just infecting one single cell. NICTABA and UDA were able to inhibit syncytium formation between persistently HIV-1-infected T cells and uninfected $CD4^+$ T cells, thus suggesting an inhibitory role of GlcNAc-binding CBAs during attachment of gp120 to cellular receptors and subsequent fusion steps. This is an important property of the CBAs that is shared with other HIV entry inhibitors (dextran sulphate (DS-5000),[68] enfuvirtide[69] or the CXCR4 antagonists[47]), but is absent in drugs that target a site in the replication cycle of HIV located at a timepoint after viral entry.

DC-SIGN is a lectin of our innate immune system predominantly present on immature DCs. It functions in DC recognition and the uptake and processing of pathogens leading to antigen presentation to T cells. It has been shown that DC-SIGN can recognize and internalize numerous bacteria, protozoa[70] and viruses such as HIV,[70] Dengue virus,[71] human CMV,[72] HCV[73] and Ebola virus[74] to allow for efficient *trans* infection of the target cells. Preventing interaction with DCs, and in particular DC-SIGN, represents an attractive approach to prevent viral transmission and infection of the host. We showed that UDA but not NICTABA efficiently prevents the DC-SIGN-directed capture of HIV-1. Interestingly, both NICTABA and UDA prevented transmission of DC-SIGN-captured HIV-1 to uninfected $CD4^+$ T-lymphocytes in the same concentration range needed for inhibition of cell-free viral transmission in T-lymphoma cell lines (EC_{50} NICTABA ~140 nM, UDA ~600 nM).

Some CBAs have been shown to be not equally effective in their inhibitory potential for both modes of transmission.[14,47] NICTABA is the first CBA reported so far that does not block DC-SIGN-directed HIV capture. This suggests that NICTABA recognizes different glycan epitopes than DC-SIGN, while UDA, at least partially, shares certain glycan epitopes present on gp120 or hinders DC-SIGN binding. Several studies have revealed that the carbohydrate-recognition domain of DC-SIGN recognizes α-(1,3)- and α-(1,2)-linked mannose oligomers[75] as well as fucosylated glycans.[76] That NICTABA prevented HIV-1 transmission but not viral capture could be explained by the assumption that its binding to the GlcNAc-containing glycans on gp120 prevents conformational changes and hinders the flexibility of gp120 that is required to properly interact with the cell membrane receptor necessary for fusion.

When taking a closer look at the results of the sugar inhibition experiments, it is clear that the binding of NICTABA and UDA to gp120 in the presence of a GlcNAc β-1,4-trimer was dose-dependently decreased. This is in line with previous reports on the GlcNAc specificity of both lectins. While this appears to be in contrast to the recent finding from a microarray screen that NICTABA also efficiently recognizes high-mannose glycans,[77] it should be mentioned that these studies found high affinity of GlcNAcβ1,4GlcNAc configurations that are extended with mannose residues. This may imply that NICTABA shows a certain affinity for mannose oligomers on the condition that they are part of a complex with GlcNAc oligomers. Generating crystal structures with $(GlcNA)_2Man_x$ oligomers might be useful to clarify this observation.

In this report, α-(1,2)-mannose and α-(1,3/1,6)-mannose trimers (devoid of GlcNAc residues) hardly influenced NICTABA binding but they did affect UDA binding to gp120, albeit to a lesser degree than in the presence of GlcNAc trisaccharides. This finding suggests that UDA has a high affinity for GlcNAc residues but, like DC-SIGN, can also recognize α-(1,3)- and α-(1,2)-linked mannose oligomers. Another observation that supports the broader sugar specificity of UDA is that binding of the monoclonal antibody 2G12 is diminished in the presence of UDA but not of NICTABA. 2G12 mAb is one of the few broadly neutralizing anti-HIV monoclonal antibodies that solely recognizes a specific configuration of three α1,2-mannose glycan oligomers on gp120, which lies around the C4-V4 region.[78-80]

2G12 mAb activity has been shown to vary depending on the nature of the HIV-1 isolates that are evaluated. Indeed, Travers *et al.*[58] has demonstrated that the 2G12 epitope is poorly conserved across the HIV-1 group M due to strong strain-specific glycosylation patterns. In this report, the observed narrow variation in the potency of NICTABA represents an interesting property with respect to the potential use for NICTABA as an antiviral agent. Furthermore, Huskens *et al.*[15] observed that under increased drug pressure from the 2G12 mAb, HIV-1 became resistant rather quickly (6 cell culture passages) and only one glycan deletion appeared sufficient for the 2G12 mAb to lose its antiviral activity. This observation was also confirmed *in vivo* where the potency of 2G12 and two other neutralizing antibodies to gp41 were evaluated in HIV-1 infected individuals.[81] Although plasma levels of 2G12 were the highest and exceeded the *in vitro* required 90% inhibitory doses, viral escape emerged very rapidly and at high titers.[81] In contrast, only after more than 90 cell culture subcultivations did a UDA-resistant virus emerged possessing 9 mutations at N-linked glycosylation sites.[25] Interestingly, more high mannose glycan-containing glycosylation sites were deleted compared with complex mannose-type glycans of gp120.[25] Complex N-linked glycans differ from the high-mannose and hybrid glycans by having added other sugars including GlcNAc residues at both the α-3 and α-6 mannose sites. Cross-resistance was observed with multiple mannose-specific CBAs (HHA, GNA, CV-N, 2G12).[25] It would be of interest to see if NICTABA-resistant virus would have a similar preference for annihilation of high mannose type N-glycans in gp120 than for complex N-linked glycans. However, after 70 subcultivations no phenotypical and genotypical resistance of HIV-1 was observed against the GlcNAc-specific NICTABA (data not shown). Unfortunately, due to a limited selectivity index (SI=21) in cell culture, we will probably not be able to increase drug pressure and thus generate an HIV-1 resistant NICTABA strain. It has been shown that certain lectins can induce a mitogenic response, for instance cyanovirin, which despite being a very potent inhibitor of viral entry, can bind to the host cell surface, induce T-cell activation markers and production of various pro-inflammatory cytokines.[82] In contrast, griffithsin one of the most potent CBAs identified to date, has also been shown to bind the host cell surface but this binding did not result in cellular activation.[82] Whether NICTABA also binds to the cell surface remains unclear, since there are currently no specific antibodies available to resolve this issue.

A better understanding of the molecular interaction between NICTABA and the GlcNAc-sugar residues on gp120 by NMR or crystallography would enable rational design of more potent synthetic GlcNAc-specific CBAs. Further research to feasibly scale-up synthetic GlcNAc-specific CBAs should be explored due to the high genetic barrier for resistance development, even more so than for mannose-specific CBAs. Another concern that is often raised for CBAs in general is their potential inability to discriminate between pathogen and cellular glycans. Nevertheless, it has been shown that the three-dimensional configuration of the glycans present on pathogens are important for its specific interaction.[14] In fact, DC-SIGN is a very good example of a lectin that can distinguish between pathogen-associated glycoproteins and cellular glycoproteins, thereby enabling a selective elimination of the pathogen. Furthermore, intravenous administration of UDA and the mannose-specific GNA and HHA to adult mice has been shown to lack toxicity.[83] In addition, for UDA, it has also been reported that at concentrations that were substantially higher than its antiviral activity in cell culture, it did not agglutinate human red blood cells and was poorly mitogenic.[25]

All things considered, targeting the envelope N-glycans of HIV and other viruses might be a promising strategy. This is also corroborated by the fact that a new generation of potent, broadly neutralizing antibodies has recently been identified from HIV-1-infected individuals that target novel epitopes on HIV gp120 which are partly or exclusively composed of glycans. [84-88] These mAbs have been shown to exhibit great breadth and antiviral potency against various HIV-1 isolates *in vitro* and showed promising results in a vaccination study in macaques.[85] These recent findings indicate that CBAs may have potential as future antiviral drugs.

In summary, NICTABA is, unlike UDA, a clearly GlcNAc-specific CBA and is endowed with a broad-spectrum antiviral activity. It may concomitantly suppress both well-known co-pathogens HIV and HSV, which is beneficial from a microbicide viewpoint.[89] Furthermore, its neutralizing activity was striking, in the sense that NICTABA displayed minimal variation in antiviral activity, irrespective of the nature of the HIV viral strain and host cell type. In addition, NICTABA was found to be a potent inhibitor of syncytium formation and of DC-SIGN directed transmission to $CD4^+$ T-lymphocytes. These properties together make NICTABA an appealing and potent candidate for further drug development against enveloped viruses. The therapeutic exploitation of a GlcNAc-specific CBA will require the design of synthetic mimics that share the same broad activity but lack the potential cellular side effects of NICTABA.

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TRANSPARENCY DECLARATIONS

None to declare.

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Table 1. Antiviral activity of NICTABA, UDA and HHA against a broad spectrum of viruses evaluated in HEL, MDCK, HeLa, Raji DC-SIGN, CrFK or Vero cell cultures.

Viruses	EC_{50}[a] (nM)		
	NICTABA[b]	UDA[b]	HHA[b]
ENVELOPED VIRUSES			
Herpes simplex virus-1 (KOS) (HEL)	263 ± 113	> 11800	> 2000
Herpes simplex virus-2 (G) (HEL)	53 ± 11	1294 ± 82	> 2000
Herpes simplex virus-1 TK^- KOS ACV (HEL)	171 ± 47.0	9647 ± 3764	> 2000
Varicella-zoster virus (HEL)	129 ± 1.32	ND	ND
Influenza A H1N1 subtype (MDCK)	32 ± 11	506 ± 153	540 ± 360
Influenza A H3N2 subtype (MDCK)	18 ± 5.3	188 ± 47	2 ± 0.2
Influenza B (MDCK)	11 ± 0.0	224 ± 176	6 ± 2
Parainfluenza-3 virus (HeLa)	50 ± 26	235 ± 0.0	> 2000
Respiratory syncytial virus (HeLa)	105 ± 0.0	> 11800	> 2000
Dengue virus Type 2 (Raji/DC-$SIGN^+$)	526 ± 2.3	1176 ± 423	12 ± 0.2
Feline Herpes virus (CrFK)	24 ± 0.3	647 ± 470	220 ± 86
Feline Corona virus (CrFK)	153 ± 1.7	1024 ± 106	520 ± 12
NON-ENVELOPED VIRUSES			
Coxsackie virus B4 (HeLa)	> 2600	> 5900	> 2000
Reovirus-1 (Vero)	> 2600	> 11800	> 2000

[a]IC_{50} (50% inhibitory concentration): the concentration to inhibit virus-induced CPE by 50%.
[b] Molecular weight of NICTABA: 38 kDa, UDA: 8.5 kDa, HHA: 50 kDa
MCC (minimal cytotoxic concentration) for NICTABA (µg/ml): HEL > 100, HeLa > 100, Vero > 100
CC_{50} (median cytotoxic concentration) for NICTABA (µg/ml): MDCK > 50, CrFK > 50, Raji/DC-$SIGN^+$ > 50
ND: not determined
Mean IC_{50} ± SEM from 3-4 independent experiments are shown

Table 2. Anti-HIV activity (EC_{50} in µM) of NICTABA, UDA and HHA against laboratory-adapted strains and clinical isolates of HIV in cell cultures and PHA-activated PBMCs

HIV-1 strains (tropism; subtype)	NICTABA	UDA	HHA
Laboratory-adapted strains			
MT-4/NL4.3 (X4; B)	0.17 ± 0.08	0.28 ± 0.08[¶]	0.004 ± 0.001[¶]
TZM-bl/BaL (R5; B)	0.23 ± 0.05	0.5 ± 0.1	0.010 ± 0.001
CEM/IIIB (X4; B)	0.023 ± 0.003	0.23 ± 0.00	0.0060 ± 0.0005
PBMCs/NL4.3 (X4; B)	0.018 ± 0.003	0.23 ± 0.06	0.005 ± 0.002
PBMCs/BaL (R5; B)	0.014 ± 0.005	0.38 ± 0.14	0.01 ± 0.004
PBMCs/HE (X4/R5; B)	0.015 ± 0.004	1.80 ± 0.02	0.240 ± 0.039
PBMCs/ROD (HIV-2)	0.005 ± 0.003	0.06 ± 0.03	ND[a]
Clinical isolates			
PBMCs/UG273 (R5; A)	0.008 ± 0.002	1.6 ± 0.7	ND[a]
PBMCs/DJ259 (R5; C)	0.020 ± 0.004	0.1 ± 0.2	0.10 ± 0.02
PBMCs/ID12 (R5; A/E)	0.010 ± 0.003	0.4 ± 0.2	ND[a]
PBMCs/ETH2220 (R5; C)	0.03 ± 0.01	0.8 ± 0.3	ND[a]
PBMCs/US2 (R5; B)	0.03 ± 0.03	ND[a]	ND[a]

[a]ND: not determined.

[¶]Data obtained from Huskens *et al.*[17]

CC_{50} for NICTABA (µg/ml): MT-4 > 20, TZM-bl > 20, CEM > 20, PBMCs: 14.4

Mean EC_{50}-values ± SEM from at least 3-5 independent experiments are shown.

Table 3. Inhibitory activity of NICTABA, UDA and HHA against syncytia formation between persistently HIV-1-infected cells and uninfected $CD4^+$ T cells.

CBA	$EC_{50}{}^{a}$ (μM)
NICTABA	0.32 ± 0.09
UDA	0.48 ± 0.1
HHA	0.08 ± 0.02

[a]EC_{50}: The effective concentration of CBA necessary to inhibit syncytia formation between persistently HIV-infected HUT-78/IIIB cells and uninfected $CD4^+$ SupT1 T cells by 50%.

Mean EC_{50}-values ± SEM of 3 independent experiments are shown.

Table 4: Inhibition of HIV-1 capture and transmission by DC-SIGN$^+$ cells to human CD4$^+$ T-lymphocytes.

CBAs	EC_{50}[a] (μM)	
	Capture	Transmission[b]
NICTABA	>1.3	0.14
UDA	0.44	0.61
HHA	0.01	0.06

[a] EC_{50}: The effective concentration of CBA necessary to inhibit HIV-1 capture by Raji DC-SIGN and the transmission of DC-SIGN-captured virus to uninfected CD4$^+$ C8166 T cells by 50%.

[b] In co-cultivation cultures with CD4$^+$ C8166 T cells, samples were taken 44 hours post co-cultivation.

Table 5: Kinetic parameters of the interaction of NICTABA and UDA with immobilised HIV-1 envelope proteins gp120 and gp41 determined through surface plasmon resonance.

HIV-1 gp 120	k_a (1/Ms)[a]	k_d (1/s)[a]	K_D (M)[a,b]
NICTABA	$1.8\ 10^{5} \pm 9.3\ 10^{1}$	$7.0\ 10^{-4} \pm 9.5\ 10^{-7}$	$3.8\ 10^{-9}$
UDA	$4.8\ 10^{5} \pm 5.7\ 10^{2}$	$4.2\ 10^{-3} \pm 3.5\ 10^{-6}$	$8.7\ 10^{-9}$
HIV-1 gp 41	k_a (1/Ms)	k_d (1/s)	K_D (M)
NICTABA	$1.7\ 10^{5} \pm 6.1\ 10^{2}$	$2.6\ 10^{-4} \pm 9.1\ 10^{-7}$	$1.5\ 10^{-9}$
UDA	$1.0\ 10^{6} \pm 2.9\ 10^{3}$	$1.6\ 10^{-3} \pm 3.3\ 10^{-6}$	$1.6\ 10^{-9}$

[a] k_a: association rate constant; k_d: dissociation rate constant; K_D: affinity constant.

[b] The affinity constant K_D is the ratio k_d/k_a.

Mean values ± SEM from 3 independent experiments are shown.

Figures

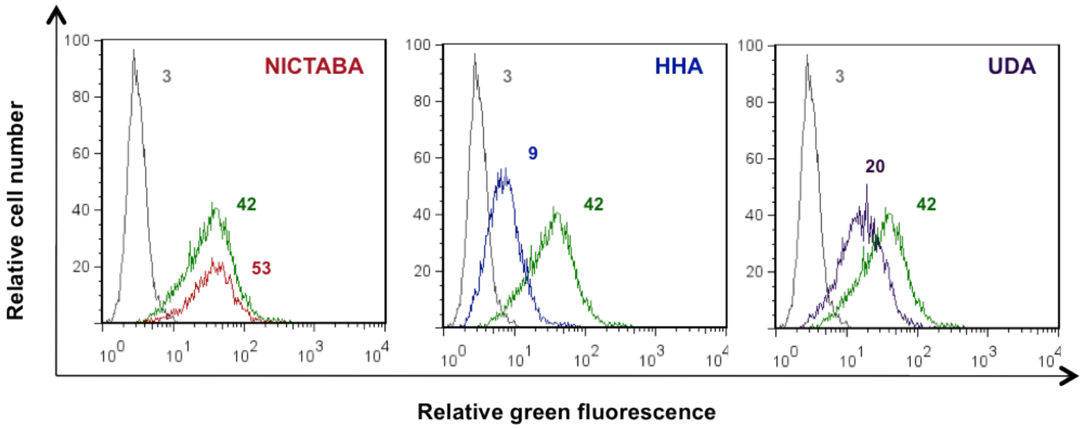

Figure 1. UDA and HHA but not NICTABA inhibit the binding of the 2G12 mAb to HIV-1 NL4.3-infected MT-4 cells.

MT-4 cells were infected with HIV-1 strain NL4.3. After 3-4 days, the cells were incubated with the 2G12 mAb (20 µg/mL) + RaH-IgG-FITC in the absence (*green curves* or presence of NICTABA (*red curves*) or HHA (*blue curves*) or UDA (*purple curves*). The *light grey curves* show the background fluorescence. The average MFI values of 3 independent experiments are indicated above each histogram and reflect the degree of binding of the 2G12 mAb to the HIV-1 infected cells. One representative experiment out of three is shown.

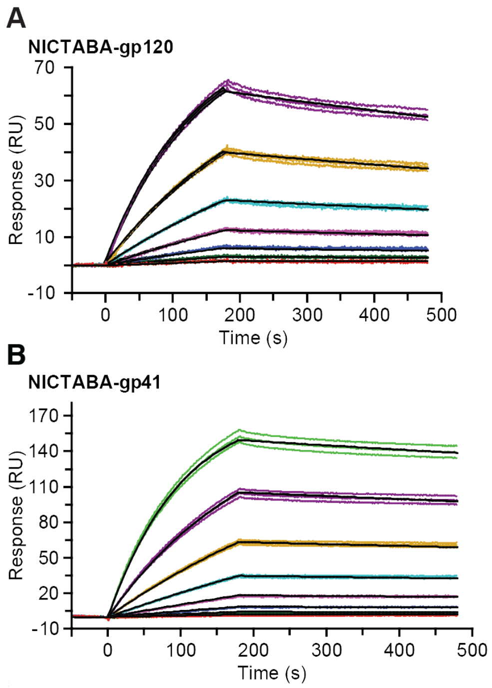

Figure 2. Kinetic analysis of CBA interactions with immobilised envelope proteins of HIV. Binding of NICTABA (left panels) or UDA (right panels) to HIV-1 IIIB gp120 **(A)** or HIV-1 HxB2 gp41 **(B)** immobilised on a CM5 sensor chip. Serial two-fold analyte dilutions were injected over the surface of the HIV-1 envelope protein-bound sensor chip. These dilutions covered a concentration range from 1.0 to 63 nM for NICTABA and from 2.0 to 63 nM (gp120) or 2.0 to 31 nM (gp41) for UDA. Experiments were carried out in triplicate (coloured lines). The curves were fitted using the 1:1 binding model (black curves) to determine the kinetic parameters.

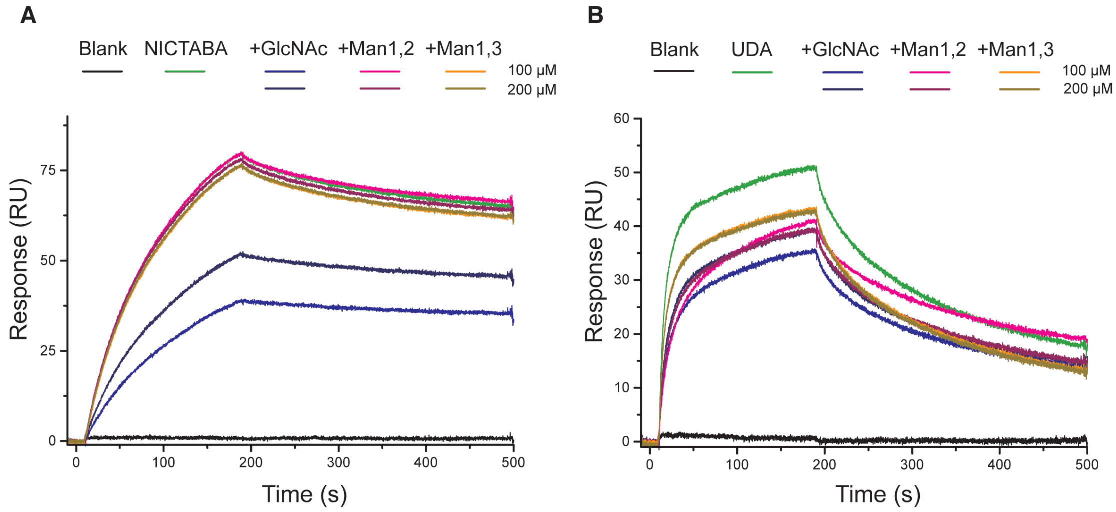

Figure 3. Affinity analysis of NICTABA (A) and UDA (B) for immobilised HIV-1 IIIB gp120 in the presence or absence of various oligosaccharides.

The concentrations of the trisaccharides (GlcNAc β-(1,4)-GlcNAc/β-(1,4)-GlcNAc; mannose α-(1,2)-mannose/α-(1,2)-mannose; mannose α-(1,3)-mannose/α-(1,6) mannose) were 200 or 100 μM. UDA concentration was kept constant at 100 nM and NICTABA at 80 nM throughout the whole experiment. The lectins, trisaccharides or a pre-incubated mixture of lectin and trisaccharide were injected across the sensor surface.